Effective Utilization of N^2-Ethyl-2′-deoxyguanosine Triphosphate during DNA Synthesis Catalyzed by Mammalian Replicative DNA Polymerases†

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ABSTRACT: Acetaldehyde is produced by metabolic oxidation of ethanol after drinking alcoholic beverages. This agent reacts with nucleosides and nucleotides, resulting in the formation of N^2-ethyl-guanine residues. N^2-ethyl-2′-deoxyguanosine (N^2-ethyl-dG) adduct has been detected in the lymphocyte DNA of alcoholic patients [Fang, J. L., and Vaca, C. E. (1997) *Carcinogenesis 18*, 627−632]. Thus, the nucleotide pool is also expected to be modified by acetaldehyde. N^2-Ethyl-2′-deoxyguanosine triphosphate (N^2-ethyl-dGTP) was chemically synthesized. The utilization of N^2-ethyl-dGTP during DNA synthesis was determined by steady-state kinetic studies. N^2-Ethyl-dGTP was efficiently incorporated opposite template dC in reactions catalyzed by mammalian DNA polymerase α and δ. When pol α was used, the insertion frequency of N^2-ethyl-dGTP was 400 times less than that of dGTP, but 320 times higher than that of 7,8-dihydro-8-oxo-2′-deoxyguanosine triphosphate (8-oxo-dGTP), an oxidative damaged nucleotide. Using pol δ, the insertion frequency of N^2-ethyl-dGTP was only 37 times less than that of dGTP. The chain extension from dC:N^2-ethyl-dG pair occurred much more rapidly: the extension frequencies for pol α and pol δ were only 3.8 times and 6.3 times, respectively, lower than that of dC:dG pair. We also found that N^2-ethyl-dG can be detected in urine samples obtained from healthy volunteers who had abstained from drinking alcohol for 1 week before urine collection. This indicates that humans are exposed constantly to acetaldehyde even without drinking alcoholic beverages. Incorporation of N^2-ethyl-dG adducts into DNA may cause mutations and may be related to the development of alcohol- and acetaldehyde-induced human cancers.

Cellular DNA can be damaged by endogenous sources and by exposure to exogenous agents. If the damage is not repaired, a misincorporation may occur opposite the modified DNA lesion during translesional synthesis catalyzed by DNA polymerases. Deoxynucleosides and deoxynucleotides can also be damaged and may be incorporated into DNA during DNA synthesis. For example, the *Escherichia coli mutT*⁻ strain, which lacks 7,8-dihydro-8-oxo-deoxyguanosine triphosphatase (8-oxo-dGTPase), showed a 500 times higher spontaneous mutation frequency than the wild-type strain (*1*). This indicates that 8-oxo-dGTP inserted into DNA causes mutation. Thus, modification of deoxynucleoside 5′-triphosphate plays an important role in mutagenesis.

Acetaldehyde is present in many foods, automotive exhaust gases (*2*), and cigarette smoke (0.8−1.4 mg is produced per cigarette) (*3*). Drinking alcoholic beverages produces acetaldehydes in the body through the metabolic oxidation of ethanol. Acetaldehyde also results from ethanol produced from carbohydrates through bacterial fermentation in the gastrointestinal tract (*4*) and is produced endogenously from sources such as threonine, β-alanine, and deoxyribose phosphate during normal intermediary metabolism (*5*, *6*). Treatment with acetaldehyde promotes adenocarcinomas and squamous-cell carcinomas in the nasal mucosa of rats and laryngeal carcinomas in hamsters (*2*). Esophageal squamous cell carcinoma is epidemiologically associated with alcohol and tobacco consumption (*7*). Human alcohol dehydrogenase gene 2 (*ADH2*) and aldehyde dehydrogenase gene 2 (*ALDH2*) genetic polymorphism is related to esophageal and liver cancer risks (*8*).

Acetaldehyde induces sister-chromatid exchanges in bone marrow cells of rodents (*9*, *10*) and in cultured human lymphocytes (*11*), chromosomal aberrations in rat embryos (*12*), and mutations in cultured human skin fibroblasts (*13*). This reagent also induces intrastrand DNA cross-links between adjacent guanine bases and tandem GG to TT base substitutions in shuttle vector plasmids propagated in human fibroblast cell lines (*14*). Acetaldehyde induces the formation of a Schiff base at exocyclic amino groups of the guanine, which can be reduced by glutathione and ascorbic acid in

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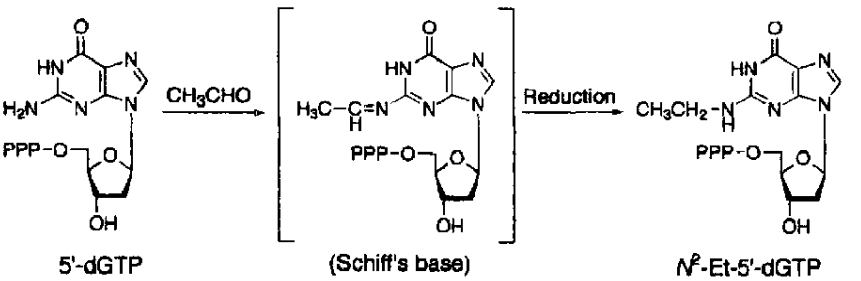

FIGURE 1: Formation of N^2-Et-dGTP through Schiff's base.

cells, to form the stable N^2-ethyl-guanine (*15*) (Figure 1). N^2-Ethyl-2′-deoxyguanosine (N^2-Et-dG)[1] adduct has been detected in liver DNA from mice treated with ethanol (*15*) and in granulocyte and lymphocyte DNA of alcoholic patients (*16*).

In this study, N^2-ethyl-2′-deoxyguanosine triphosphate (N^2-Et-dGTP) was prepared and used to determine if it can be utilized during DNA synthesis catalyzed by mammalian replicative DNA polymerases. We found that N^2-Et-dGTP was efficiently incorporated into DNA. In addition, N^2-Et-dG can be detected in human urine samples obtained from healthy nonalcohol drinkers and nonsmokers.

MATERIALS AND METHODS

Materials. Organic chemicals used for the synthesis of oligodeoxynucleotides were supplied by Ardrich. [γ-^{32}P]ATP (specific activity > 6000 Ci/mmol) was obtained from Amersham Corp. dNTPs were from Pharmacia; T4 polynucreotide kinase was from Stratagene; calf thymus DNA pol α (30,000 units/mg protein) and human pol β (100 000 units/mg) were purchased from Molecular Biology Resources, Inc. Pol δ (0.1 μg/μL) was purified from calf thymus as described by Downey and So (*17*) except that DEAE-Sephadex chromatography was omitted.

Instruments. A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of the N^2-Et-dGTP. Mass spectrometry was performed on a Krotos MS 890, equipped with a Hewlett-Packard 5980 detector. NMR spectroscopy was performed on a Bruker 600 MHz instrument. Urinary N^2-Et-dG was analyzed by the electrospray liquid chromatography tandem mass spectrometer (ESI LC/MS/MS). The LC was a Ultrafast Micro-protein Analyzer (Microm BioResources). The mass spectrometer and the data system used were a TSQ 7000 Triple-Stage Quadrupole (Finnigan MAT, San Jose) with an electrospray source and a DEC 3000 (Digital Equipment Corp., Bedford, MA), respectively. The instrument was operated in positive ion mode. A gas sheath flow of 60 psi (483 kPa) nitrogen, an electrospray voltage of 4.5 kV, and a capillary temperature of 200 °C were used. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 2.0 mTorr (0.27 Pa). The collision offset was +20 V.

Synthesis of N^2-Et-dGTP and N^2-Et-dG-d_4. To prepare N^2-Et-dGTP, dGTP (3 mg) was reacted overnight with 0.1 mL of acetaldehyde in 1 mL of 0.2 mM sodium acetate buffer (pH 4.5) and reduced by $NaBaH_4$ (Figure 1). N^2-Et-dGTP was purified on a reversed-phase μBondapak C_{18} column (0.39 × 30 cm, Waters), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 0 to 50% acetonitrile, with an elution time of 40 min and a flow rate of 1.0 mL/min. N^2-Et-dGTP was identified by using mass spectroscopy and ^{1}H NMR. To measure N^2-Et-dG in human urine using ESI LC/MS/MS, N^2-Et-dG and N^2-Et-dG-d_4 were synthesized as similarily described by Vaca et al. (*15*) and used as an internal standard. N^2-Et-dG-d_4 was purified on a reversed-phase μBondapak C_{18} column (0.39 × 30 cm, Waters), using a linear gradient of 20 to 90% methanol in water, with an elution time of 20 min and a flow rate of 1.0 mL/min. Purity of the N^2-Et-dG-d_4 was confirmed by UV spectrum and a mass spectroscopy. (*N^2-Et-dG*) ^{1}H NMR: δ 10.49 (brs, H1), 7.89 (s, C8), 6.35 (t, 1H, J = 5.2 Hz, N^{2}H), 6.15 (t, 1′−H), 5.28 (d, J = 4.0 Hz, 3′-OH), 4.86 (t, 5′-OH), 4.36 (m, 3′−H), 3.81 (m, 4′−H), 3.50 and 3.56 (m, 5′−H), 3.30 (m, $-CH_2CH_3$), 2.20 and 2.64 (m, 2′−H), 1.14 (t, $-CH_2CH_3$, J = 7.2 Hz). (*N^2-Et-dGTP*) ^{1}H NMR: δ 10.46 (brs, H1), 7.91 (s, C8), 6.37 (brs, 1H, N^{2}H), 6.16 (t, 1′−H), 5.39 (brs, 3′-OH), 4.49 (m, 3′−H), 3.95−3.82 (m, 3H, 4′−H and 5′−H), 3.30 (m, $-CH_2CH_3$), 2.16 and 2.66 (m, 2′−H), 1.14 (t, $-CH_2CH_3$, J = 7.2 Hz). Me_2SO-d_6 solution was measured with tetramethylsilane as an internal standard.

Synthesis of Oligodeoxynucleotides. DNA templates (5′-CCTTC*N*CTTCTTTCCTCTCCCTTT, *N*=C, A, G, or T) and primers (5′AGAGGAAAGAAG, and 5′AGAGGAAAGAAGG) were prepared by solid-state synthesis on an automated DNA synthesizer. A 13-mer primer (5′AGAGGAAAGAAGX, X = N^2-Et-dG) containing N^2-Et-dG at the 3′-terminus was prepared by enzymatic incorporation of N^2-Et-dGTP using the 12-mer primed template (5′AGAGGAAAGAAG/5′-CCTTTCCTTCTTTCCTCTCCCTTT) and *E. coli* exo$^-$ Klenow fragment and purified on a denaturing polyacrylamide gel.

Primer Extension Reactions. A 24-mer template (0.75 pmol, 5′CCTTC*N*CTTCTTTCCTCTCCCTTT) primed with a ^{32}P-labeled 12-mer (0.5 pmol, 5′AGAGGAAAGAAG) was used for the determination of N^2-Et-dGTP or dGTP insertion opposite the dC, dA, dG, or dT embedded in template. The amounts of N^2-Et-dGTP or dGTP incorporation were measured at 25 °C in 10 μL of a buffer containing DNA polymerase and either N^2-Et-dGTP or dGTP. A ^{32}P-labeled

[1] Abbreviations: N^2-Et-dG, N^2-ethyl-2′-deoxyguanosine; dGTP, 2′-deoxyguanosine triphosphate; N^2-Et-dGTP, N^2-ethyl-2′-deoxyguanosine triphosphate; pol α, DNA polymerase α; pol β, DNA polymerase β; pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; ESI LC/MS/MS, electrospray liquid chromatography tandem mass spectrometer.

13-mer primer (0.5 pmol, 5′AGAGGAAAGAAGX, X = N^2-Et-dG or dG) was used for the determination of the chain extension. The amounts of chain extension were measured at 25 °C in the presence of dGTP. The buffer for pol α consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and BSA (0.5 μg/μL). The buffer for pol δ consisted of 50 mM Tris-HCl, pH 6.5, 10 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, BSA (0.04 μg/μL), and PCNA (6 ng/μL). Reactions were stopped by adding formamide dye and heating to 95 °C for 3 min. The reaction samples were subjected to 20% PAGE containing 7 M urea.

Steady-State Kinetic Study. Steady-state kinetic parameters were established for N^2-Et-dGTP or dGTP insertion opposite dN in templates and for chain extension from dN:N^2-Et-dG or dC:dG pair from the 3′ primer terminus using pol α or pol δ. Reaction mixtures containing 0.05−0.3 unit of pol α or 0.15 unit of pol δ, varying amounts of N^2-Et-dGTP or dGTP, and 1.0 pmol of 24-mer template primed with 0.5 pmol of ^{32}P-labeled 12-mer in 10 μL of the buffer were used to measure nucleotide insertion. Reaction mixtures containing 1.0 pmol of 24-mer template primed 0.5 pmol of ^{32}P-labeled 13-mer containing N^2-Et-dGMP or dGMP at the 3′-terminus, and varying amounts of dGTP were used to measure chain extension. Reaction mixtures were subjected to electrophoresis on 20% polyacrylamide gels (35 × 42 × 0.04 cm) in the presence of 7 M urea. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were obtained from Hanes−Woolf plots of the kinetic data. Frequencies of insertion (F_{ins}) and extension (F_{ext}) were determined relative to the dC:dG base pair according to the equation $F = (V_{max}/K_m)_{(\text{wrong pair})}/(V_{max}/K_m)_{(\text{correct pair=dC:dG})}$.

Analysis of N^2-Et-dG in Human Urine. Human urine samples were obtained from six healthy nonalcohol drinking and nonsmoking individuals. The concentration of creatinine in the urine samples was measured by "Creatinine Test Kit Wako" (Wako, Japan). The pH of urine was adjusted to 7.0 by the addition of HCl or NaOH. The urine was then centrifuged, and 200 ng of N^2-Et-dG-d_4 was added to 10 mL of the supernatant. The supernatant was then applied to the Sep-Pak Plus C_{18} Environmental Cartridge (Waters). The cartridge was washed with 10 mL of water and eluted with 5 mL of methanol. The eluant was then allowed to evaporate to dryness, the residue was redissolved with 200 μL of water, and 600 μL of cold ethanol was added to precipitate proteins. After centrifugation, the supernatant was evaporated to dryness and redissolved with 200 μL of water. The sample was then fractionated by HPLC on a reversed-phase Wakosil II 5C18 RS column (1.0 × 30 cm, Wako), using a linear gradient of 10 to 70% methanol in water, with an elution time of 30 min and a flow rate of 2.0 mL/min. N^2-Et-dG was collected according to the retention time of synthetic standards. The collected fraction was dried and then redissolved in 200 μL of water and subjected to ESI LC/MS/MS analysis. The sample was analyzed with a reversed-phase MONITOR C_{18} column (0.1 × 15 cm, Microm BioResources) attached to a trap column. Solvents used were as follows: solvent A, 0.04% of acetic acid and 5 mM of ammonium acetate in water; solvent B, 50% acetonitlile (v/v), 0.04% acetic acid, and 5 mM ammonium acetate in water. The HPLC gradient system was as follows: 0 to 5 min, isocratic, solvent A 98%, solvent B 2%; 5 to 40 min, a linear gradient of 2 to 98% of the solvent B and 98 to 2% of solvent A. A selected reaction monitoring (SRM) was performed on both N^2-Et-dG (*m/z* 296→180) and N^2-Et-dG-d_4 (*m/z* 300→184). The detection limit of N^2-Et-dG was 0.05 pmol (15 pg).

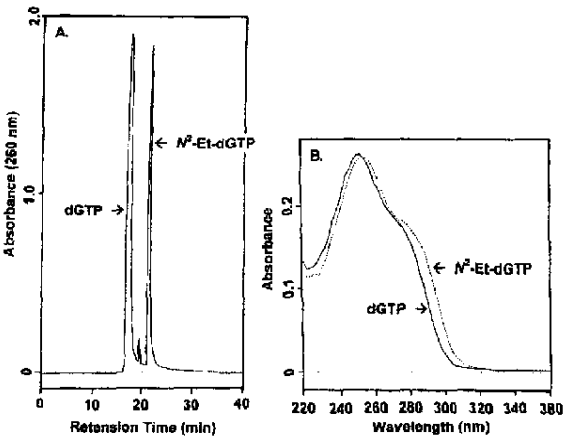

FIGURE 2: HPLC separation of N^2-Et-dGTP. (A) dGTP was reacted with acetaldehyde in sodium acetate buffer, pH 4.5, and reduced by $NaBaH_4$ as described in the Material and Methods. N^2-Et-dGTP was isolated from dGTP on a reversed-phase μBondapak C_{18} (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 0 → 50% acetonitrile over 40 min and a flow rate of 1.0 mL/min. (B) UV spectra of N^2-Et-dGTP and dGTP.

RESULTS

Preparation of N^2-Et-dGTP. When dGTP was reacted with acetaldehyde and reduced by $NaBaH_4$, N^2-Et-dGTP (t_R = 21.1 min) was isolated completely from dGTP (t_R = 17.1 min) by HPLC (Figure 2A). UV spectrum of N^2-Et-dGTP was shifted slightly to longer wavelength as compared with that of dGTP (Figure 2B). With the use of negative ion FAB-mass spectroscopy, the parent ion exhibited at *m/z* 534 representing 535 Da. This molecular ion gave daughter ions at *m/z* 505 (M − ethyl-H)$^-$, 454 (M − H_2PO_3)$^-$, and 177 (M − deoxyribosyl triphosphate-H)$^-$. The ^{1}H NMR data of N^2-Et-dGTP were quite similar to that of N^2-Et-dG, as described in the Materials and Methods. The triplet of the 5′-OH of deoxyribose at δ 4.86 was detected with N^2-Et-dG, but not with N^2-Et-dGTP. This indicates that the 5′-OH was phosphorylated. Thus, N^2-Et-dGTP was identified by mass spectroscopy and ^{1}H NMR.

Incorporation of N^2-Et-dGTP in Reactions Catalyzed by DNA Polymerase. Using steady-state kinetic studies, the frequency of incorporation of N^2-Et-dGTP or dGTP opposite dN (dC, dA, dG or dT) embedded in templates was measured by using mammlian replicative DNA polymerases, pol α and pol δ (Table 1). Using pol α, preferential incorporation of N^2-Et-dGTP opposite template dC, the correct base, was observed. K_m and V_{max} values were obtained from Hanes−Woolf plots (Figure 3A). The insertion frequency (F_{ins}) of N^2-Et-dGTP (2.5×10^{-3}) was 400 times lower than that of dGTP. No F_{ins} was dectected opposite other dNs, although small amounts of incorporation of N^2-Et-dGTP were observed opposite template dG and dA when incubated for 20 min or 1 h (Figure 4). When pol δ was used, N^2-Et-dGTP was inserted only opposite template dC. F_{ins} (2.7×10^{-2}) of

Table 1: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reaction Catalyzed by pol α[a]

	Insertion			Extension			
	dXTP ↓GAAGAAAGGAGA[32]P 5'CCTTCNCTTC TTT CCTCTCCCTTT			dGTP ↓XGAAGAAAGGAGA[32]P 5'CCTTCNCTTC TTT CCTCTCCCTTT			
N:X	K_m (μM)	V_{max} (%min^{-1})	F_{ins}	K_m (μM)	V_{max} (%min^{-1})	F_{ext}	$F_{ins}xF_{ext}$
Pol α							
C:G	0.40±0.15	147±16	1.0	5.6±1.2	81±5.4	1.0	1.0
C:N^2-Et-dG	44±10	40±0.2	2.5×10^{-3}	8.8±2.1	33±2.6	0.26	6.5×10^{-4}
Pol δ							
C:G	2.3±0.5	59±8.0	1.0	1.8±0.75	36±5.2	1.0	1.0
C:N^2-Et-dG	27±9.0	19±2.3	2.7×10^{-2}	4.0±1.4	13±2.7	0.16	4.4×10^{-3}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in the Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair)}$. X = dG or N^2-Et-dG. [b] Data were expressed as means ± SD.

Table 2: Concentration of N^2-Et-dG in Human Urine

			N^2-Et-dG	
sample	age	sex	(nmol/L urine)	(μmol/mol creatinine)
1	28	F	0.052	0.013
2	24	F	0.072	0.010
3	24	F	0.055	0.015
4	21	F	0.052	0.010
5	30	M	0.064	0.008
6	28	M	0.057	0.015
			0.059 ± 0.008[a]	0.012 ± 0.003[a]

[a] Average values.

N^2-Et-dGTP opposite template dC was 37-fold lower than that for dGTP (Table 1 and Figure 3B). No incorporation of N^2-Et-dGTP opposite the other dNs was observed even when incubated for 1 h (Figure 4). Thus, F_{ins} of N^2-Et-dGTP opposite template dC with pol δ was 11-fold higher than that with pol α.

Chain Extension of N^2-Et-dGMP in Reactions Catalyzed by DNA Polymerases. Using 24-mer templates primed with a ^{32}P-labeled 13-mer containing N^2-Et-dGMP at the 3′-terminus, chain extension reactions catalyzed by pol α or pol δ were carried out in the presence of dGTP (Table 1 and Figure 3). Chain extension reaction from the dC:N^2-Et-dG pair occurred rapidly by all the polymerases tested. The extension frequency (F_{ext}) from the dC:N^2-Et-dGMP was 0.26 for pol α and 0.16 for pol δ. The overall frequency ($F_{ins} \times F_{ext}$) for dC:N^2-Et-dG pair for pol δ (4.4×10^{-3}) was 6.8-times higher than that for pol α (6.5×10^{-4}).

Presence of N^2-Et-dG in Human Urine. Human urine samples were obtained from healthy volunteers who were nonalcohol drinkers and nonsmokers. The volunteers had consumed alcoholic beverages for at least 1 week before the urine collection. Urine samples were pretreated with a Sep-Pak Plus C_{18} cartridge and a HPLC for enrichment of N^2-Et-dG and analyzed by ESI LC/MS/MS. N^2-Et-dG-d_4 was added to the urine samples as an internal standard. A selected reaction monitoring (SRM) was performed on both N^2-Et-dG and N^2-Et-dG-d_4. N^2-Et-dG was detected as a clear peak at the same retention time as N^2-Et-dG-d_4 (Figure 5). The amount of N^2-Et-dG was calculated by the ratio of peak areas of N^2-Et-dG and N^2-Et-dG-d_4. N^2-Et-dG was detected in all urine samples tested, and the average concentration was 0.059 nmol/L of urine (0.012 mmol/mol of creatinine) (Table 2).

DISCUSSION

N^2-Et-dG excreted in urine may be formed as follows: (a) acetaldehyde directly reacts with dG to form N^2-Et-dG, (b) N^2-Et-modified nucleotides produced by acetaldehyde, including N^2-Et-dGTP, are decomposed enzymatically to N^2-Et-dG in cells, and (c) N^2-Et-dG lesions formed by incorporation of N^2-Et-dGTP into DNA or by reacting DNA directly with acetaldehyde may be excised by DNA repair process. 7,8-Dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) has been used as a biomarker of oxidative DNA damage (*18*). The amounts of 8-oxo-dG in human urine could be useful as an index of the risk of the oxidative damage (*19,20*). Similarly, N^2-Et-dG in human urine may be used as a biomarker to evaluate acetaldehyde-induced damage in human body.

N^2-Et-dG was detected in urine of healthy nonsmokers who had abstained from alcohol for 1 week (Table 2). Acetaldehyde is found in many foods and is produced from endogenous sources such as threonine, β-alanine, and deoxyribose phosphate (*5, 6*). Since alcohol is added to many foods as a food additive and is also produced from carbohydrates through bacterial fermentation in the gastrointestinal tract (*4*), acetaldehyde can be produced by the metabolic oxidation of alcohol. These acetaldehyde sources may result in the formation of N^2-Et-dG in the body. This indicates that humans are constantly exposed by acetaldehyde even without taking alcohol beverages.

N^2-Et-dGTP was preferentially incorporated opposite template dC during DNA synthesis. The insertion frequency

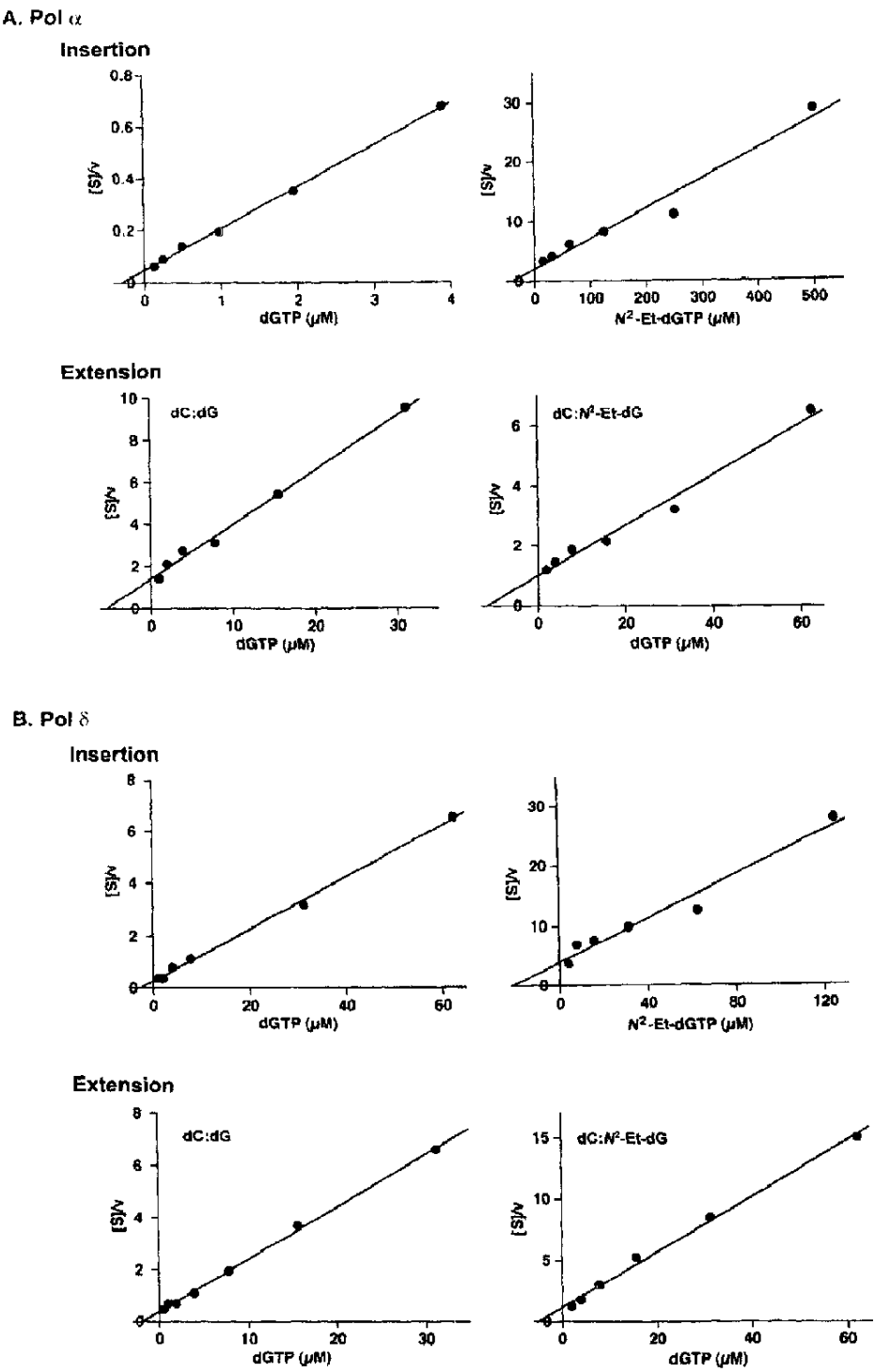

FIGURE 3: Hanes–Woolf plots of N^2-Et-dGTP or dGTP insertion and the chain extension. Using pol α (A) or pol δ (B), the K_m and V_{max} values of dGTP or N^2-Et-dGTP insertion opposite template dC and chain extension of dC:dG or dC:N^2-Et-dG pair were obtained from Hanes–Woolf plots, as described in the Materials and Methods.

(F_{ins}) of N^2-Et-dGTP was 400 times lower than that of dGTP with pol α (Table 1). Using pol δ, F_{ins} of N^2-Et-dGTP was 37 times less than that of dGTP. Thus, pol δ incorporated N^2-Et-dGTP into DNA 11 times higher than pol α. The insertion frequency of N^2-Et-dGTP varies depending on DNA polymerase used. In addition, the frequencies of chain

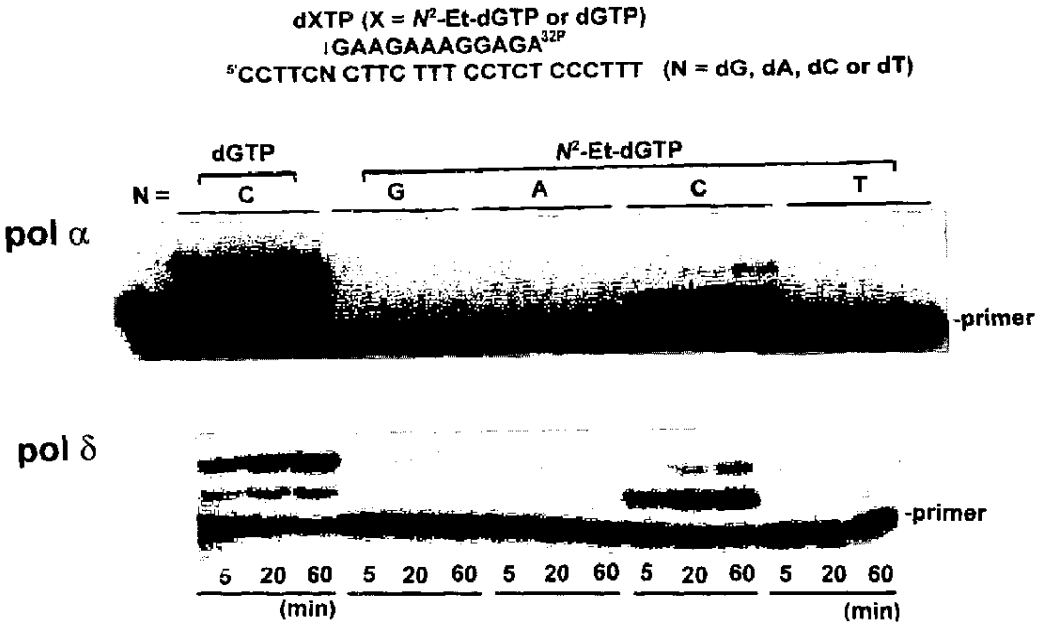

FIGURE 4: Time course of N^2-Et-dGTP or dGTP insertion on the DNA template. Using a 24-mer template (5'CCTTC*N*CTTCTTTC-CTCTCCCTTT, N = C, A, G, or T) primed with a ^{32}P-labeled 12-mer (5'AGAGGAAAGAAG), incorporation of N^2-Et-dGTP or dGTP opposite the dC, dA, dG, or dT embedded in template was measured at 25 °C using 1.2 unit of pol α or 0.3 unit of pol δ, as described in the Materials and Methods.

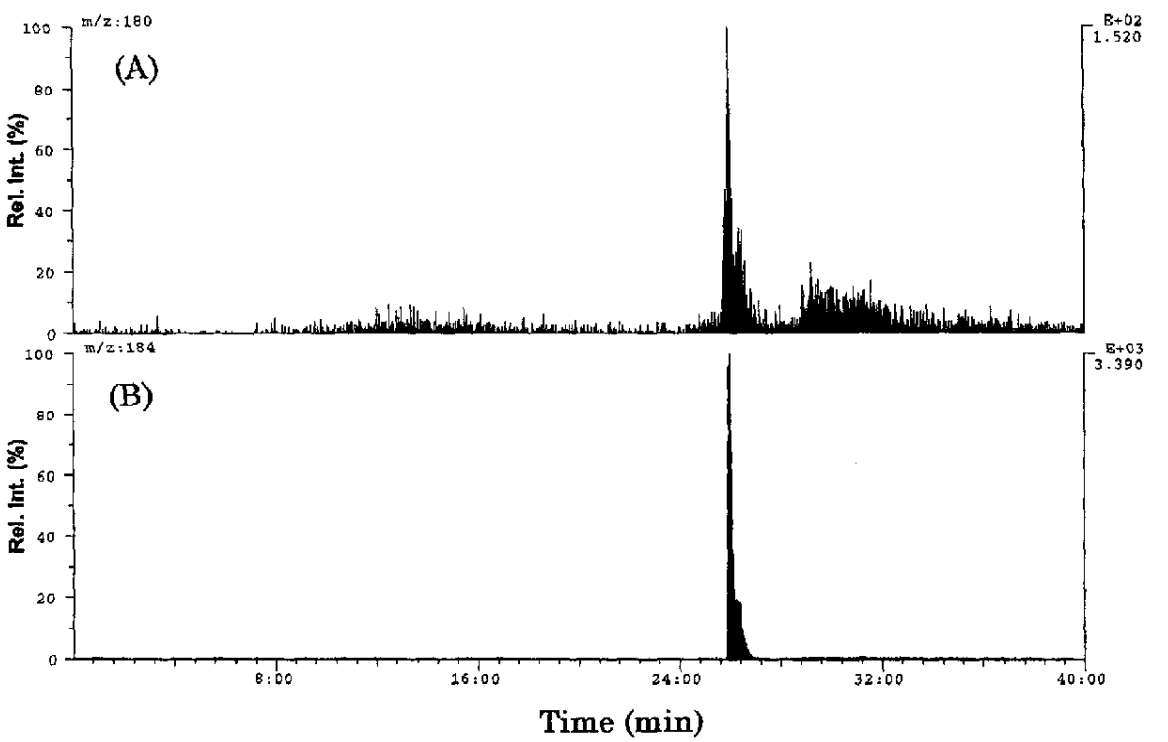

FIGURE 5: Detection of N^2-Et-dG in human urine. A human urine sample containing N^2-Et-dG-d_4 as an internal standard was analyzed by ESI/LC/MS/MS. The analytical condition was as described in Materials and Methods. Both N^2-Et-dG (target) and N^2-Et-dG-d_4 (internal standard) were analyzed by dual selected reaction monitoring (SRM) of (A) m/z 296 → 180 for target N^2-Et-dG and (B) m/z 300 → 184 for internal standard N^2-Et-dG-d_4.

extension (F_{ext}) from dC:N^2-Et-dG pair were only 3.8 times lower for pol α and 6.3 times lower for pol δ, respectively, than that of dC:dG pair. The chain extension from dC:N^2-Et-dG occurred much more rapidly than the insertion of N^2-

Table 3: Comparison of Insertion Frequency of N^2-Et-dGTP with 8-oxo-dGTP

dN:dXTP	F_{ins}	ratio
pol α		
dC:N^2-Et-dGTP	2.5×10^{-3}	320
dC:8-Oxo-dGTP[a]	7.7×10^{-6}	1
pol β		
dC:N^2-Et-dGTP	1.7×10^{-2}	6.7
dC:8-Oxo-dGTP[a]	2.6×10^{-3}	1

[a] Data were taken from Loeb et al. (*21*).

Et-dGTP opposite template dC. Thus, N^2-Et-dGTP may be efficiently incorporated into DNA during DNA replication in living cells.

Insertion frequency of N^2-Et-dGTP was compared with that reported previously for 8-oxo-dGTP using pol α and pol β (*21*) (Table 3). Similar insertion frequency of 8-oxo-dGTP was also observed with pol β by our colleague (*22*). When pol α was used, F_{ins} for N^2-Et-dGTP opposite dC was 320 times higher than that for 8-oxo-dGTP. With pol β, F_{ins} for N^2-Et-dGTP opposite dC (1.7×10^{-2})[2] was 6.7 times higher than that for 8-oxo-dGTP. Thus, utilization of N^2-Et-dGTP during DNA synthesis may be much higher than 8-oxo-dGTP.

The concentration of N^2-Et-dG in urine was 0.012 mmol/mol of creatinine. This value is at least 110 times lower than that of urinal 8-oxo-dG reported by several groups: 1.36 mmol/mol of creatinine (*19*) and 1.42−2.27 mmol/mol of creatinine (*20*). Although 8-oxo-dG DNA lesions can be repaired by 8-oxo-guanine DNA glycosylase (*23*), in this case, the 8-oxo-guanine base, not 8-oxo-dG, is excised. Since a human homologue of MutT has been shown to exist (*1*), a part of 8-oxo-dG detected in human urine may result from the decomposition of 8-oxo-dGTP by 8-oxo-dGTPase. This process may contribute to increase 8-oxo-dG in urine. Although the repair process for N^2-Et-dGTP and N^2-Et-dG DNA lesion has not yet been investigated, drinking alcohol beverages may increase the level of N^2-Et-dG in urine.

Small amounts of misincorporation of N^2-Et-dGTP were observed opposite template dG when incubated for 20 and 60 min with pol α (Figure 4). We also explore nucleotide incorporation opposite N^2-Et-dG lesion embedded in a DNA template, using our established in vitro experimental system (*24*, *25*): pol α promoted a small amount of dGMP misincorporation.[2] Both misincorporation of N^2-Et-dGTP opposite template dG and dGTP opposite template N^2-Et-dG may generate G → C transversions in mammalian cells. The mutational spectrum was consistent with that of acetaldehyde-induced mutations observed in human cells (*14*). Thus, N^2-Et-dG lesions formed by efficient incorporation of N^2-Et-dGTP into DNA and by reacting DNA with acetaldehyde may cause mutations and may be involved in the development of alcohol- or acetaldehyde-induced cancers.

[2] I. Terashima and S. Shibutani, unpublished data.

Although N^2-Et-dG DNA adducts have been detected in liver of mice treated with ethanol (*15*) and in white blood cells of alcoholic patients (*16*), the level of nucleotide modified by acetaldehyde has so far not been determined in mammalian tissues. Further studies will be required to explore the carcinogenicity of acetaldehyde.

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